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TITLE

Synthesis and evaluation of new cyanine dyes as minor groove binders

DESCRIPTION

Technical field

The present invention relates to new cyanine dyes particularly suited for use in DNA sequencing.

Background of the invention

The introduction of combinatorial chemistry, the sequencing of the human genome and miniaturisation, e.g. lab-on a chip, nanochemistry, has enabled the creation of vast libraries of "new chemical entities", millions of which must be quickly tested by high-throughput screening to identify active sites and drugs. Drugs that bind reversible to DNA in the minor groove of DNA has been synthesised with the aim to generate new lead compounds with anticancer and antiviral properties. Formerly, radioactive probes have been used to study the effect of drug-DNA interactions but during the last years they have started to be replaced by different fluorogenic assays. Today, drug-DNA interactions are mainly studied with absorbance spectroscopy, fluorescence dye displacements assays, footprinting or NMR. Since the numbers of fluorescence markers are limited to a few there is a challenge to discover new fluorescent dyes that circumvent the limitations on those that now are available. New fluorogenic compounds that bind in the minor groove can either work in dye displacement assays or give insight in how substituents may work as minor groove recognition elements.

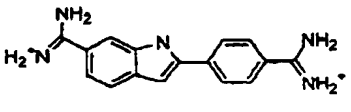
Fluorogenic compounds can provide tremendous sensitivity due to large quantum emission yield upon excitation. A limitation is that there are not many fluorophores that give a high increase in fluorescence upon hybridisation or reaction with targets.

Asymmetric cyanine dyes have achieved much interest recently due to their excellent properties as non-covalent labels for nucleic acids. Upon binding to nucleic acids these dyes exhibit a very large enhancement in fluoresence intensity and have been used as fluorescent markers for DNA in various contexts. This class of cyanine dyes have been used in various applications as fluorescent markers for DNA, *e.g.* flow cytometry, [1]restriction fragment

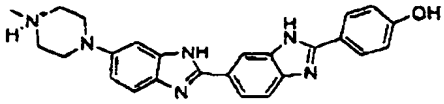
sizing, as reporter groups in hybridisation probes, gel-staining, and in real time PCR. The interaction between the monomeric dyes TO and YO and DNA have been investigated spectroscopically in a variety of studies. These dyes binds by intercalation between the bases in a non-specific fashion. Furthermore they also have a strong affinity for single stranded DNA with a large accompanying increase in fluorescence upon binding.

The fluorophores that are most frequently used today are Fluorescein, BODIPY, DAPI, Hoechst and asymmetric cyanine dyes such as TO, YO and TOTO.

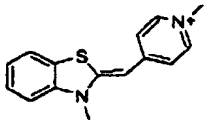
Fluorescein and BODIPY are the most common fluorescent reporter groups for covalent labeling of proteins whereas DAPI, Hoechst and Cyanine dyes are the most common fluorophores for detection of nucleic acid.



DAPI



A HOECHST DERIVATE



A CYANINE DYE

DAPI (abs. max 400 nm) and Hoechst (abs. max 350 nm) bind in the minor groove and are used as base-specific fluorescent probes for DNA with a 20-fold increase in fluorescence upon binding to DNA.[2] In contrast, asymmetric cyanine dyes has shown up to a 18.000-fold increase in fluorescence upon binding to DNA. They also have the advantage that the absorption and emission can be easily varied by changing the number of double bounds between the aromatic rings. However, a major drawback with asymmetric cyanine dyes is that they usually bind in a non-specific fashion towards DNA-sequences.[3] (i.e. intercalate or form ion-pair complexes to DNA which may result in complex or weak fluorescence signal.) Therefore a cyanine dye that bind in a more organised way may have high fluorescence increase upon hybridisation and thus, be a more sensitive fluorophores.

The minor groove is a convenient site for attack since it is normally unoccupied by cellular compounds such as proteins. It is also a perfect complement to concave cationic dyes due to the negative electrostatic potential and the convex floor of the minor groove. Certain minor groove binders stabilise DNA duplexes and can work as regulators of DNA-protein function.

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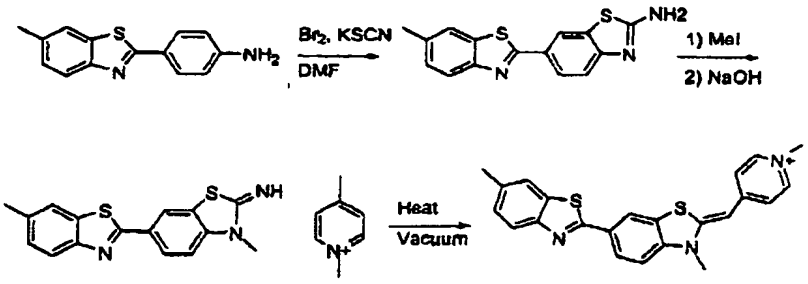
[2] Biochem Biophys Res comm 170, 270 (1990)
[3] Nygren J., Svanvik, N., Kubista, M.Biopolymers, 46, 39-51, 1998

As a consequence, the development of sequence-specific minor groove binders may generate new compounds with anticancer and/or antiviral properties and thus, serve as an alternative and complementary approach to the antisense oligonucleotide strategy. Furthermore, the minor groove binders stabilising effect upon DNA duplexes can be used in probes, consisting of a minor-groove ligand-nucleic acid conjugate, to increase the melting temperatures (Tm) of probe-DNA duplexes. An increase of the Tm of probes will allow a more flexible assay design since the oligo in the probe can be shorter and still have an optimal Tm.

Sequence selective minor groove binders also has mismatch discrimination. Nucleic acid probes with minor groove binders as reporter group should have an increased difference between the Tm of match and single-base mismatch nucleic acids than the corresponding probe with an intercalator as reporter group. Thereby increasing the discriminatory power of hybridisation assays.

A useful feature of minor groove binders are a preference for double stranded DNA compared to single stranded DNA whereas intercalators usually has no preference for single or double stranded DNA. This feature result in that minor groove binder probes will have lower background fluorescence than probes with an intercalator and as a consequence, a greater signal-to-noise ratio upon hybridisation. Furthermore, dyes specific for duplex-DNA can be used for quantification of DNA in mixtures contaminated by RNA or single stranded DNA.

One challenge is to develop numbers of highly sensitive fluorescent dyes with different well-separated emission spectra that bind in a precise way and thus allowing multidetection of a serie of targets with high sensitivity. As mentioned, cyanine dyes can have up to a 18.000-fold increase in fluorescence upon hybridisation which is almost 1000 times higher than the minor groove binders that are used today. Also the absorption and emission are easily tuned by varying the conjugated system in cyanine dyes. Thus, a cyanine dye substituted so that binding in the minor groove is govern but with the extraordinary fluorescence properties of the known cyanine dyes retained seems to be a highly interesting target compound.



Inspired by the concave structure of minor groove binders and the new findings that a benzothiazole and relatedly structured groups may govern minor groove recognition[4] we have designed and synthesised an asymmetric cyanine dye substituted with an extra benzothiazole group in accordance with above.

The interaction between this new dye and DNA were studied with various spectroscopic methods such as flow-LD and CD.

These two techniques can provide information on whether a drug is binding to DNA by intercalation or groove binding. Weak induction of CD is usually associated with intercalating whereas asymmetric induction is due to groove binding. Groove binding give a strong signal in Flow-LD.

In the presence of calf thymus DNA a weak positive signal was observed in the flow LD-spectra. This can be due to heterogeneous binding with a mixture of intercalated and groove binding dye. On the other hand, in the presence of poly $[(dA\text{-}dT)]_2$ a clear positive LD is shown providing a strong indication of minor groove binding. For poly $[(dG\text{-}dC)]_2$ only a weak negative signal was observed indicating a heterogeneous binding or a low abundance of intercalated dye.

Clearly the new dye binds differently to A-T rich and G-C rich regions. Results from CD-measurements gave further support for groove binding of this new dye.

[4] Armitage et. al. J. Am. Chem. Soc. 121, 2987-2995, (1999)

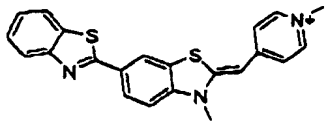
For poly GC almost no signal is seen which is consistent with intercalative or external binding, whereas for poly AT a very strong asymmetric induction is seen.

It binds to the minor groove of A-T rich regions and thus it stabilises A-T bonds more than G-C bonds in a DNA duplex. Therefore, if a probe is designed so that an A-T rich region is placed under the minor groove binder it can be used in probes to improve mismatch discrimination.

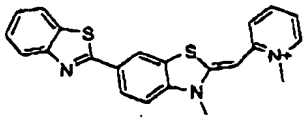
Interestingly, our results further accentuate the preliminary reports in the literature that the benzothiazole group has utility as a minor groove recognition element. If so, this is an important finding, since its opens possibilities for design of new drugs binding in the minor groove.

Our first results show that it is possible to design and prepare asymmetric cyanine dyes that work as minor groove binders.

Further, possibilities of broadening the present scope are: Since there is a well working synthetic route for the substituted cyanine dye the first step is the nitrogen in ortho position, **2-BEBO**, from the methine substituent.

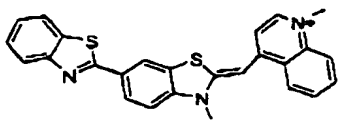


BEBO

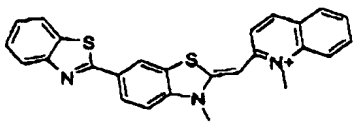


2-BEBO

Along with the synthesis of the two quinolinium derivates, **BETO** and **2-BETO**, the synthesis of the benzoxazole and benzimidazole derivates can be done.

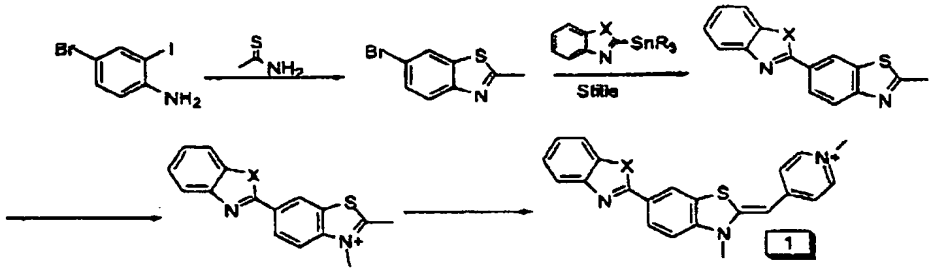


BETO



2-BETO

The synthesis of these new benzoxazole and benzimidazole substituted dyes will follow a slightly different synthetic route.

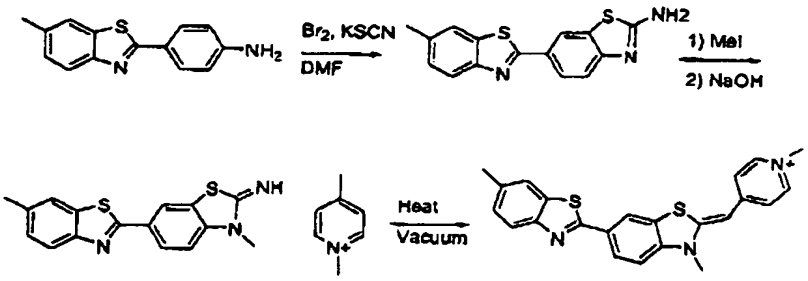


The Stille coupling of similar compounds and the synthesis of the benzooxazole- and benzimidazole-stannanes can be found in the literature.[5] The last step, the condensation of compound 1 with the pyridinium or quinolinium salt are routinely used in the synthesis of asymmetric cyanine dyes.

[5] Kosugi et. al. Bull. Chem. Soc. Jpn. 59, 677-679 (1986), Jutzi et. al. J. Organomet. Chem. 246, 163-168 (1983), Benhida et. al. Tetr. Lett. 40, 5701-5703 (1999)

Example

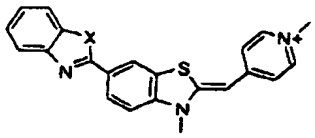
Preparation according to the reaction scheme



The dye **1** was prepared in four steps starting from the commercially available aniline **1**. Thiocyanation of the 4-substituted aniline **1** with potassium thiocyanate and bromine in DMF afforded the 2-aminobenzothiazole **2** in a 40 % yield. Methylation and deprotonation of compound **2** proceeded in a total 70 % yield to produce the 2-imino-3-methylbenzothiazoline **3**. The dye **5** was prepared in 20 % by melting compound **3** together with the pyridinium salt **4** at 160 °C under vacuum.

CLAIMS.

1. A cyanine dye binding in the groove of DNA, selected from the group of



wherein X is O, S, or N.

2. A cyanine dye according to claim 1, having the pyridine ring in 2-position.

3. Probe for nucleic acid hybridization comprising a cyanine dye according to claims 1-2.

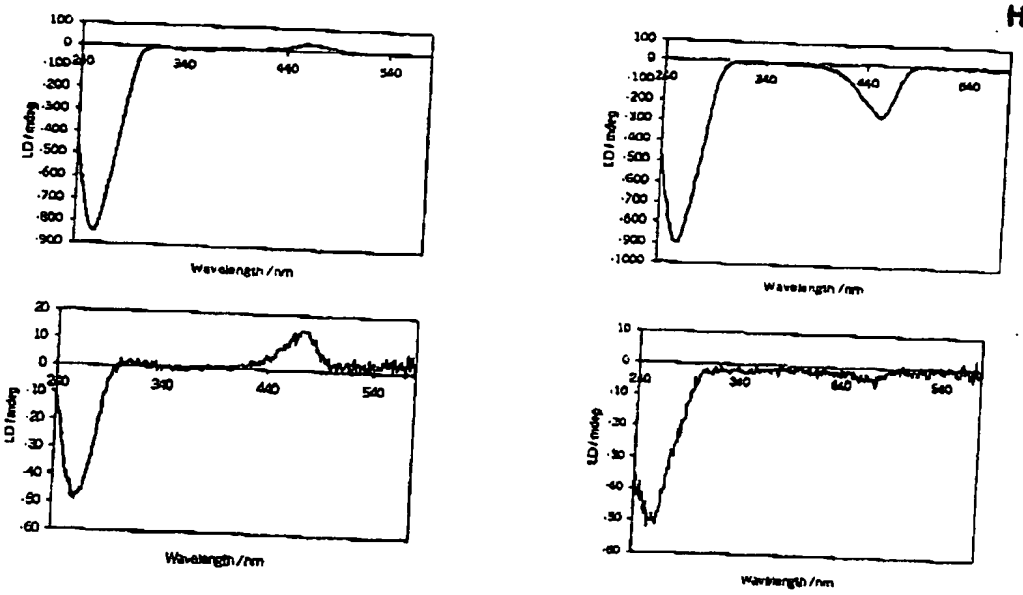


Figure 1. Flow LD spectra of BEBO complexed with: calf thymus DNA (top left), poly[dA-dT]$_2$ (bottom left), poly [dG-dC]$_2$ (bottom right), and BO complexed with calf thymus (top right). Mixing ratios (R = dye / DNA bases) were 0.05 in all cases except for poly [dG-dC]$_2$ (R = 0.02).

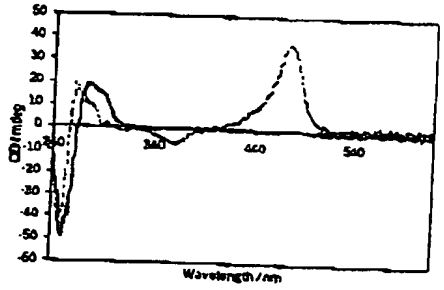
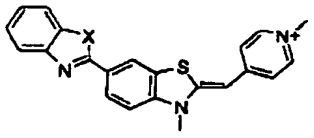


Figure 2. CD spectra of BEBO complexed with: (- - -) poly[dA-dT]$_2$, (——) poly [dG-dC]$_2$, at R = 0.05 and R = 0.02, respectively.

ABSTRACT

The present invention relates to new cyanine dyes according to the formula



wherein X is O, S, or N.